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Corrigendum

Axon guidance effects of classical morphogens Shh and BMP7 in the hypothalamo-pituitary system

Fang Liu[a,b,*], Marysia Placzek[b]

[a] School of Basic Medical Sciences, Medical College of Nanchang University, Nanchang, Jiangxi 330006, PR China
[b] MRC Centre for Developmental and Biomedical Genetics and Department of Biomedical Science, University of Sheffield, Sheffield S10 2TN, UK

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ABSTRACT

The hypothalamus plays a key role in homeostasis. Many of its effector functions are mediated through neuroendocrine neurons whose axons project to the median eminence or posterior pituitary. Understanding the guidance of hypothalamic neuroendocrine axons in development therefore adds important insight into hypothalamic function. Previous studies show that FGF10 deriving from the medial ventral midline of the hypothalamus plays an important role in attracting developing neuroendocrine axons. Here we show that Shh and BMP7, which are expressed in the anterior and posterior hypothalamic ventral midline respectively, together repel hypothalamic axons towards the medial ventral midline.

1. Introduction

In the developing nervous system, axon guidance depends on extracellular matrix cues deriving from cells along the pathway, and on gradients of chemoattractant/chemorepellant molecules emanating from more distant cellular sources [6,9,39]. Since the 1990s, Netrins, Slits, Semaphorins and Ephrins have been identified as four major conserved axon guidance cues [9,33]. However, these four classical families of guidance cues cannot explain all guidance events. In addition to these four major conserved families of guidance cues, other molecules have been implicated in axon guidance. In particular, families of morphogens have been reported to play an important role in regulating axon guidance. Morphogens are secreted proteins produced by restricted groups of cells, and provide positional information through the establishment of long-range concentration gradients [15,32,38]. Recently, morphogens with evolutionarily-conserved roles in patterning embryonic tissues, such as Hedgehogs (Hhs), bone morphogenetic proteins (BMPs), Wnts and fibroblast growth factors (FGFs) have been shown to act as guidance cues [5,31]. The patterning function of such morphogens begins at very early stages of development, so it is reasonable to speculate that a temporally-sustained gradient of these morphogens might provide directional information for axons along body axes at later stages [41]. Here, our studies are consistent with the emerging idea that classical morphogens are potent axon guidance cues, demonstrating that Shh and BMP7 can act as guidance cues to direct hypothalamic neurosecretory axons into the medial ventral midline (MVM) of the hypothalamus through repulsion.

2. Materials and methods

2.1. In situ hybridization

Embryos were processed for in situ hybridization as described previously [36]. Following development, embryos or explants (minimum 5 in each condition) were analysed as whole-mount preparations or cryostat sections.

2.2. Explant culture

All embryos were staged and dispase-isolated (1 mg/ml, Roche). According to the requirements of distinct experiments, different parts of the hypothalamus were dissected out. Explants were then cultured in collagen beds based on published techniques [28]. When two explants were cultured together, the distance between them was around 100–300 μm.

2.3. Tracing of neural projections with DiI

The lipophilic carbocyanine dye, Di-I (Molecular Probes) was injected into the MVM in open-book preparations, explants cultured to E6 and fixed in 4% paraformaldehyde.

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* Corresponding author at: Medical College of Nanchang University, School of Basic Medical Sciences, Nanchang, Jiangxi 330006, PR China.
E-mail addresses: mdp06fl@hotmail.com (F. Liu), m.placzek@sheffield.ac.uk (M. Placzek).

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2.4. Protein use

Proteins (BMP7, Shh, and chordin) were obtained and used as described previously [22]. Proteins were presented in one of two ways: on beads, or by adding directly to the culture medium. Proteins (BMP7, Shh) were pre-soaked on Affigel beads (Pharmacia Biotech). In co-cultures, beads were positioned approximately 300 μm from the explants. In blocking experiments, Chordin or the Shh blocking antibody, 5E1 (20 μg/ml; [10]) were added to the culture medium at the start of incubation.

2.5. Immunofluorescence analyses

Immunohistochemical analysis of explants was performed according to standard whole-mount or cryostat sectioning techniques [24]. Antibody anti-Neurofilament (3A10; DSHB) was used. Secondary antibodies were conjugated to Alexa 594 or Alexa 488 (Molecular Probes).

2.6. Statistical analyses

Axon numbers were recorded after 48 h, and data were analysed using Graph Pad Prism 4.0 for PC. Statistical significance of differences in means between groups was determined using the students paired *t*-test or unpaired *t*-test. *P* values less than 0.05 were considered significant for our analyses.

3. Results

3.1. Shh and BMP7 expression during the innervation of the developing hypothalamus

As described previously, hypothalamic neuroendocrine axons respond to guidance cues at E4-E5, and project to the medial ventral midline (MVM) (the forming infundibulum/neurohypophysis) at E6 [16]. We first characterized the expression of *Shh* and *BMP7* at E5. In contrast to its usual restricted midline expression elsewhere along the neuraxis, *Shh* is expressed in ventricular zone/subventricular zone (VZ/SVZ) cells in and around the anterior ventral midline (AVM) and basal plate of the hypothalamus (Fig. 1A–C, G). *BMP7* has been reported to be expressed quite widely in the ventral midline of the hypothalamus at early stages (E2–E3) [18,22]. Our results reveal that by E5, *BMP7* is more confined and expressed only in and around VZ/SVZ cells of the posterior ventral midline (PVM) (Fig. 1D–G).

To further analyse the growth of axons, we performed retrograde Di-I-labelling experiments ($n = 5$) into 'open book' hypothalamic explants. Di-I, the lipophilic carbocyanine dye,

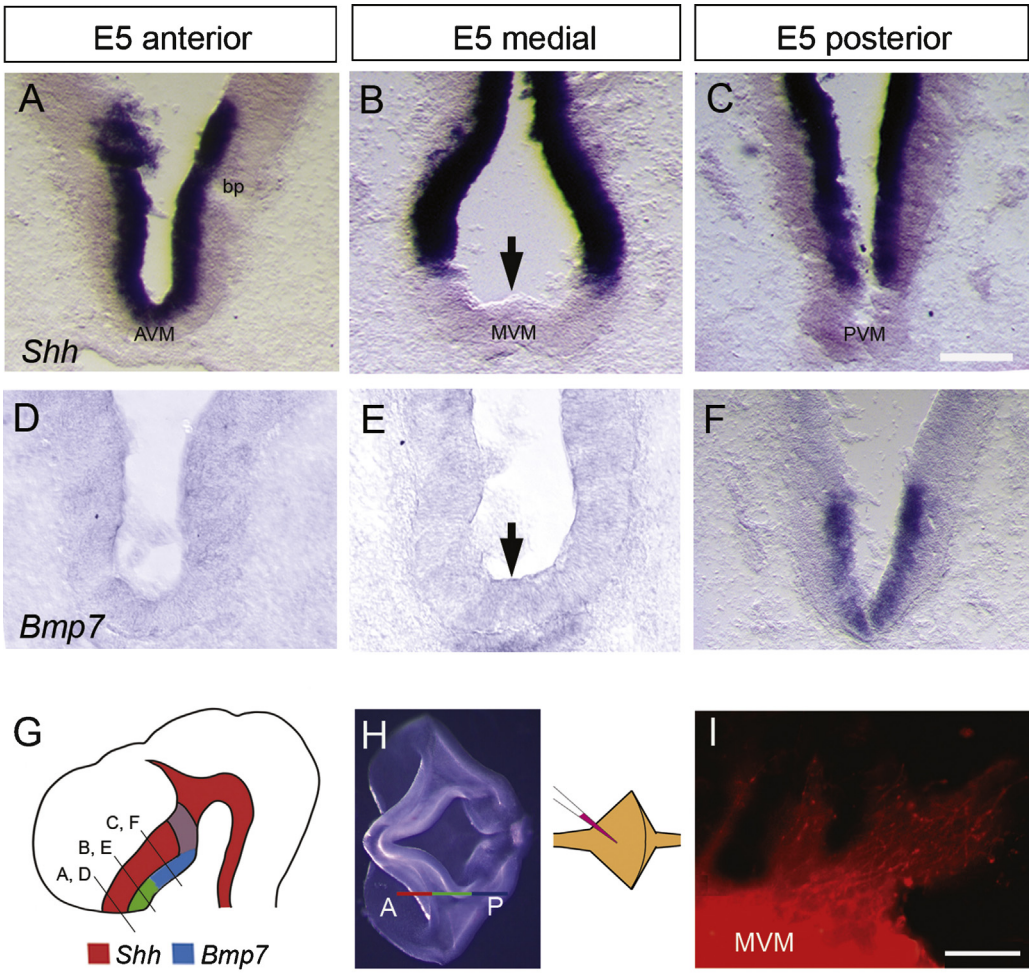

Fig. 1. (A–F) In situ hybridization on transverse sections at E5. (A–C) *Shh* is expressed in the ventricular zone/sub ventricular zone (VZ/SVZ) in the basal plate (bp) and in the anterior hypothalamus, including cells in and around the anterior ventral midline (AVM); *Shh* is not expressed in the medial or the posterior ventral midline (arrow in B). (D–F) *BMP7* is detected in VZ/SVZ cells in and around the posterior ventral midline (PVM), while there is no obvious expression in the anterior or medial hypothalamus, including the medial ventral midline (MVM: arrow in E). (G) Cartoon showing expression profiles of morphogens at E5; planes of section in (A–F) indicated. (H) Ventral view of dissected E4 hypothalamus: anterior, medial and posterior regions can be distinguished morphologically; cartoon shows Di-I targeting into the medial ventral midline. (I) Retrograde labeling shows that axons of hypothalamic neurons have entered into the medial ventral midline. Abbreviations: A, anterior; P, posterior. Scale bar: 100 μm.

dissolves in the lipid layer of the plasma membrane and diffuses anterogradely and retrogradely along neuronal processes [12]. The hypothalamus was dissected out in an 'open-book' flat configuration, so that anterior, medial and posterior portions of the ventral midline could be distinguished morphologically (Fig. 1H left-hand panel; [24]). Di-I was injected into the MVM domain of the explants (schematised in Fig. 1H right hand panel), and explants were cultured for 2 days, until the equivalent of E6. Long axons/fascicles project into the MVM (Fig. 1I).

3.2. Chemorepulsive effects of AVM and PVM

Throughout the posterior neural axis, the floor plate is known to play an important role in guiding spinal axons [6]. Studies have shown that the ventral midline of the hypothalamus shares an origin with floor plate cells of the hindbrain [18,23], which raises the possibility that the ventral midline of the hypothalamus might also influence the growth of hypothalamic axons. Previous studies, indeed, have shown that in the developing hypothalamus, neuroendocrine axons follow a diffusible chemoattractive cue(s) secreted by the MVM to reach their target cells [30] and have identified FGFs as this chemoattractive cue [16].

To directly address whether other regions of the hypothalamic ventral midline can guide axons, we performed 3-d collagen co-cultures, a well-established method to detect long-range chemotropic and chemorepulsive activities [29,34]. The hypothalamic ventral midline (and immediately adjacent cells) was subdissected, to isolate 'AVM', 'PVM' and, for comparison, 'MVM' explants (Fig. 2A: note explants contain ventral midline and immediately adjacent cells). Such explants were then cultured at a short distance from lateral hypothalamic explants that contain developing neuroendocrine neuronal cell bodies (Fig. 2A; [16]) for 2 days. Very few axons emerge from lateral explants cultured alone (Table 1, Fig. 2B). By contrast, robust axon outgrowth is elicited by the midline explants (Fig. 2C, F, I). In marked contrast to the robust attraction provoked by MVM explants ($n = 5$; Fig. 2F, H; [16]), AVM and PVM explants both mediate a repulsion of hypothalamic axons (Fig. 2C, E, I, K) ($n = 5,11$ respectively), with significantly more axons projecting away from either PVM or AVM explants (Table 1). Thus, explants expressing *Shh* and *BMP7* (Fig. 2D, J) repel hypothalamic axons.

Together, these results suggest that *Shh*-expressing cells in and around the AVM and *BMP7*-expressing cells in and around the PVM repel hypothalamic axons, in contrast to the chemoattractive effects of *FGF10*-expressing cells in the MVM [16].

3.3. Shh/BMP7 mediate the repulsive effects of the ventral midline

Morphogens can contribute to axonal pathfinding in various systems [5,31]. Our previous studies have shown that FGF10 deriving from the MVM of the chick hypothalamus plays an important role in guiding hypothalamic axons to turn into the

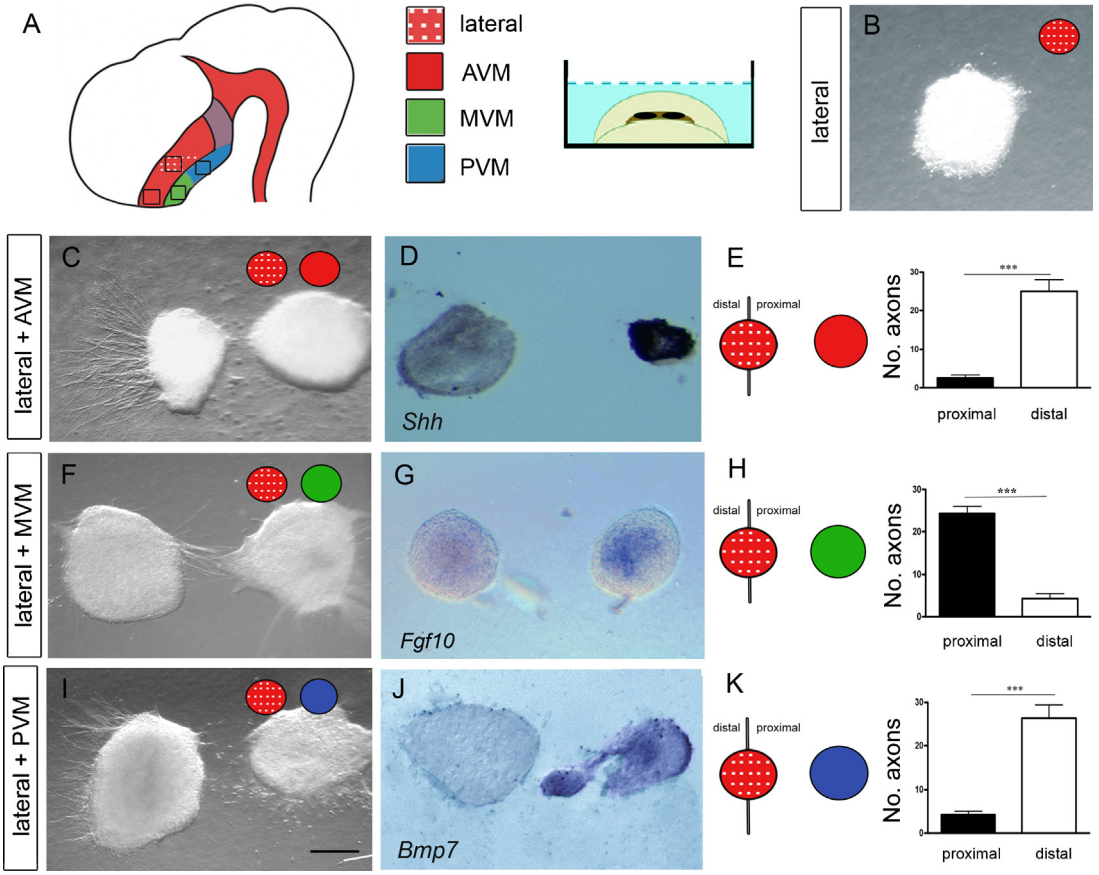

Fig. 2. AVM and PVM hypothalamic explants repel hypothalamic axons.
(A) Schematic, showing regions subdissected to obtain AVM, MVM, PVM and lateral explants and positioning in collagen gel. (B) Minimal numbers of axons emerge when lateral explants are cultured alone. (C, F, I) Co-culture of AVM (C), MVM (F) or PVM (I) with lateral explants shows that the ventral midline explants promote extensive axon outgrowth; axons appear to be repelled by PVM and AVM, in contrast to the attractive effects of the MVM. (D,G,J) In situ hybridization on similar explants to those shown in (C,F,I) confirms accuracy of dissection. (E, H, K) Significantly more axons extend from the distal face versus the proximal face of lateral explants cultured with AVM or PVM explants (***$P < 0.001$). Significantly more axons extend from the proximal versus the distal face of lateral explants cultured with MVM explants [16]. Error bars represent s.e.m. Scale bar: 100 μm.

Table 1
Statistical analyses between groups and statistical analyses between axons in different regions (proximal and distal). The number of axon bundles (mean ± SEM) are shown. Statistical comparisons between groups (co-cultured groups versus LE cultured alone group) and statistical comparisons between axons in different regions (proximal versus distal) are also shown. SEM = standard error of the mean, LE = lateral explant, n = number of lateral explants analysed, T (1) = unpaired t-test score, T (2) = paired t-test score. Number of axon bundles in LE cultured alone group is: 4.182 ± 1.354.

Groups	n	Number of axon bundles		vs LE cultured alone		Proximal vs Distal	
		Proximal	Distal	T (1)	*P*	T (2)	*P*
LE + PVM	11	4.273 ± 0.7273	26.36 ± 3.043	6.989	<0.0001	8.302	<0.0001
LE + BMP7 bead	7	6.857 ± 1.438	26.14 ± 1.908	11.78	<0.0001	7.586	0.0003
LE + BMP7 bead + chordin	5	4.200 ± 0.9695	5.400 ± 0.8718	2.327	0.0355	1.809	0.1447
LE + AVM	5	2.600 ± 0.6782	25.00 ± 3.033	8.459	<0.0001	6.766	0.0025
LE + Shh bead	7	2.429 ± 1.020	17.29 ± 3.205*	4.628	0.0003	4.934	0.0026
LE + Shh bead + 5E1	7	4.000 ± 1.091	4.286 ± 0.9689	1.913	0.0738	0.2289	0.8265

* shows comparison between LE + BMP7 bead (section ii) and LE + Shh bead (section ii) (P = 0.0351, T = 2.375; T = unpaired t-test score).

FGF10-expressingF MVM at E4–6 [16]. Our analyses suggest that BMP7 and Shh, both of which display morphogen activity in the early embryo [26] are expressed in and around, respectively, the PVM and AVM of the chick hypothalamus at E4–6, which raises the possibility that the morphogens BMP7 and Shh might be responsible for the repulsive effects of these regions of the hypothalamic ventral midline.

To establish whether Shh might be a repellant cue for hypothalamic axons, lateral explants were dissected out from E4 embryos, and then cultured with Shh-soaked beads in collagen gels. Like the AVM of the hypothalamus, Shh-soaked beads repel hypothalamic axons in vitro ($n = 7$) (Fig. 3A, E; Table 1). Moreover, the Shh blocking antibody 5E1 effectively blocks the chemorepellant function of Shh ($n = 7$; Fig. 3C, G; Table 1). These observations indicate that Shh deriving from cells in and around the AVM of the hypothalamus, and, potentially, the basal plate, may act as a repellant guidance cue for hypothalamic neurosecretory axons.

We next examined the response of lateral explants from E4 chick embryos to a BMP7 gradient, the source of BMP7 provided by an Affigel bead loaded with recombinant protein. BMP7 beads elicit robust outgrowth of hypothalamic axons that grow away from the BMP7 source ($n = 7$) (Fig. 3B, F; Table 1). Chordin is a BMP inhibitor whose function is to bind to extracellular BMP ligands and prevent the interaction between the ligands and their receptors [8,27].

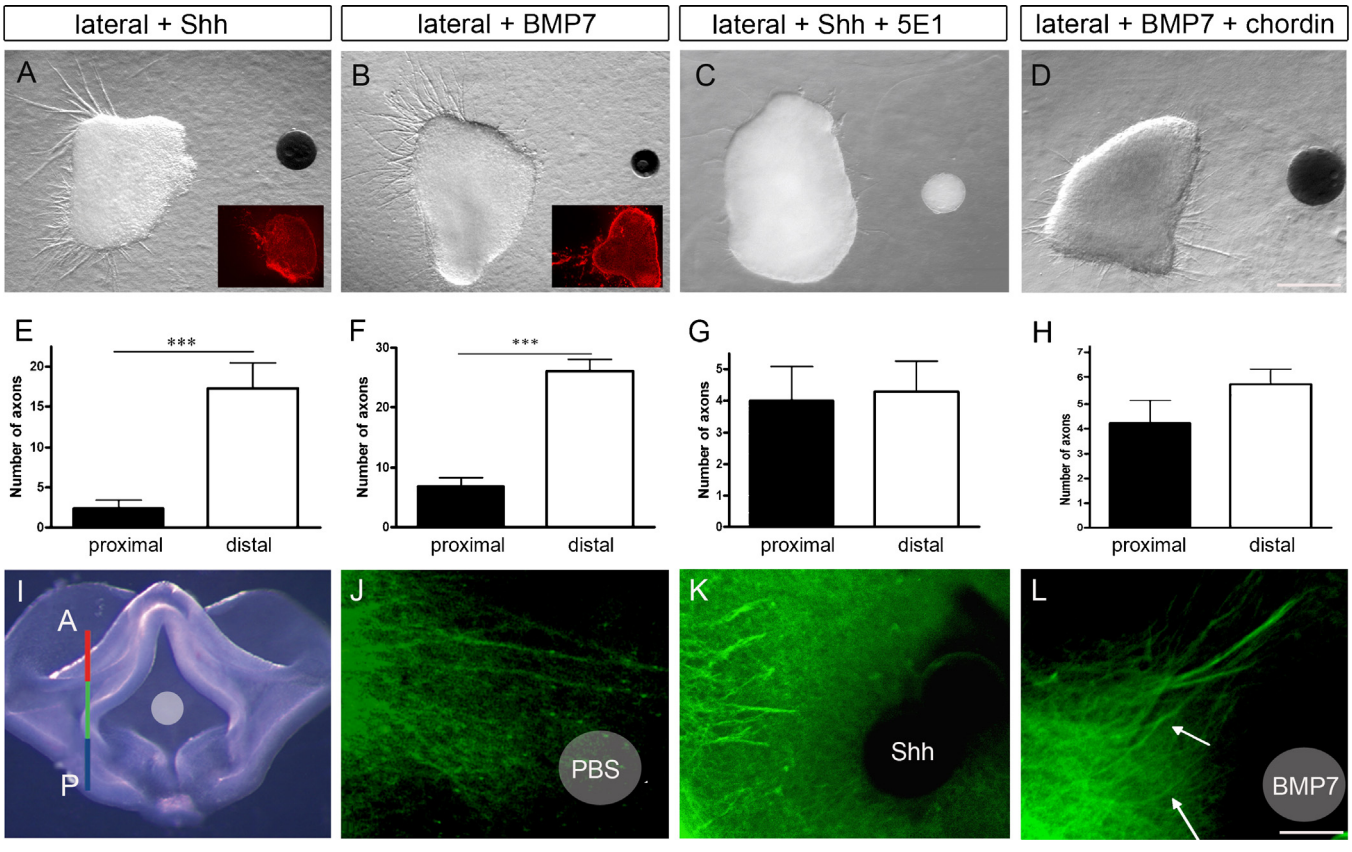

Fig. 3. Shh and BMP7 repel and orient hypothalamic axons.
(A,B) Shh and BMP7 beads repel hypothalamic axons. (C, D) Treatment with 5E1 or with chordin abolishes the axon repulsive effect. (E–H) Quantitative analyses show that the number of axons in distal sections after culture with Shh (E) or BMP7 (F) is significantly more than the number of axons in proximal sections; however, there is no statistical difference between axons in distal and in proximal sections in the presence of 5E1 (G) or chordin (H). Inset is A, B shows neurofilament (3A10) expression in axons in, and projecting from, lateral explants cultured with Shh or BMP7 beads. Scale bar: 100 μm. (I–L) 'Open-book' hypothalamic explants with beads implanted into MVM (schematic shown in I) and cultured under control conditions (PBS bead; (J) or with ectopic Shh (K) or BMP7 (L) beads. After culture, axons are examined through 3A10 expression. (J) In the control group, hypothalamic axons grow into the MVM. (K) With Shh beads implanted in the MVM, hypothalamic axons are stalled in the lateral hypothalamus/basal plate. (L) With BMP7 beads in the MVM, hypothalamic axons (arrows, L) turn away from the MVM. Scale bar: 50 μm.

In the presence of chordin, the axon-repellant activity promoted by BMP7 is blocked ($n = 5$; 7) (Fig. 3D, H; Table 1). Similarly, the repellant activity of the PVM is eliminated (not shown). This result supports our hypothesis that BMP7 acts as a chemorepellant for hypothalamic axons.

Expression of the mature neuronal marker Neurofilament (3A10) was then examined on lateral explants ($n = 5$ explants each). As remarked in other studies, when lateral explants were cultured alone, or in the presence of PBS-soaked beads, axons remain confined to the explant: axons extend to the edge of the lateral explant, then circle around the peripheral component, instead of extending into the collagen gel (not shown; [16,34]). However, in the presence of Shh or BMP7 beads, axons emerge from the explants, and strong immunolabelling of Neurofilament is detected, both on cells/axons within the lateral explant, and on axons that extend into the collagen (Fig. 3A, B inset). These results indicate that the Shh and BMP7-soaked beads exert a long-range effect on hypothalamic axons.

To further examine evidence for a chemorepellant effect of BMP7 and Shh on endogenous hypothalamic axons, we implanted Shh or BMP7 beads ectopically into the MVM of E4 'open book' hypothalamic explants (Fig. 3I), and cultured these for 2 days. In the control group (with a PBS bead implanted in the MVM) (Fig. 3J), hypothalamic axons, labelled by 3A10 anti-Neurofilament antibody, all project into the MVM. By contrast, when Shh-soaked beads are implanted in the MVM, the extension of hypothalamic axons is prevented at a defined distance from the MVM and axons fail to project beyond the lateral hypothalamus/basal plate (Fig. 3K). A different effect is provoked by BMP7 beads: here, a large portion of axons avoid the MVM by turning away from it (Fig. 3L). These data suggest that ectopic Shh and BMP7 affect the projection of endogenous hypothalamic axons, albeit, potentially, through different mechanisms. Together, these analyses suggest a requirement for Shh and BMP7 in early hypothalamic axon guidance.

4. Discussion

The hypothalamo-pituitary neuraxis is established when neuroendocrine neurons located in distinct nuclei in the hypothalamus project axons, via the MVM, that eventually synapse with capillaries in either the median eminence or the neural-derived posterior pituitary (both derivatives of the MVM) [19,25,40]. Accuracy of projection of hypothalamic axons is therefore pivotal to ensure that neurosecretory neurons connect with the endocrine system. Given this, surprisingly little is known of the mechanisms that govern axon guidance in this region. Here, our observations add to the growing body of evidence that BMP7 and Shh, besides their well characterized effects as morphogens, have additional functions as axon guidance cues.

4.1. Roles of Shh and BMP7 in the chemorepulsion of hypothalamic axons

Shh and BMPs have long been reported as morphogens that regulate the dorso-ventral patterning of the neural tube [13,14]. However, at later stages, the remaining gradient of BMPs and Shh establishes the ventral trajectory of commissural axons in the spinal cord [1,3,4]. In the chick hypothalamus, BMPs and Shh collaborate to play key roles in regulating cell identities [7,18,21,22]. Here we show that, as in the spinal cord, the remaining gradients of BMP7 and Shh also contribute to axon guidance in the hypothalamus. Increasing evidence indicates that both BMPs and Shh can function as either chemattractants or chemorepellants [1,2,4,11,17,20,35]. A number of lines of evidence suggest that BMP7 and Shh might act as chemorepellants for hypothalamic neurosecretory axons. First, both promote an outgrowth of axons in vitro that is directed down the concentration gradient of Shh/BMP7. Second, antagonists of BMP and Shh signalling, chordin and 5E1, respectively, can block such axon guidance effects of BMP7 and Shh. Third, chordin can negate the repellant activities of the hypothalamic posterior ventral midline. Finally, in a situation that more closely matches the in vivo environment, ectopic BMP7 and Shh can interfere with the correct pathway of endogenous hypothalamic axons. The activity of BMPs and Shh in specifying cell identities in the hypothalamus raises the possibility that the guidance effects of BMP7 and Shh are not due to a chemorepellant function, but due instead to their morphogenic effects. However, our ectopic BMP7/Shh experiments go some way towards proving a direct effect of BMP7 and Shh on hypothalamic axons. Moreover, in the chick, BMPs govern the patterning of the medial hypothalamus at relative early stages (before E5) [7,18,21,22]. Interestingly, recent studies have shown that the re-orientation of axons by BMP7 or Shh is distinct from their ability to induce distinct neurons. Shh and BMP7 were found to act directly on developing axons without evoking transcription within neuron cells [35,37]. In sum, these data strongly suggest chemorepulsive effects of BMP7 and Shh on hypothalamic axons.

Our in vitro experiments suggest that BMP7 and Shh act directly on hypothalamic axons to repel these axons into the median eminence. However, there are differences between the chemorepulsive effects of BMP7 and Shh. Our data indicate that more individual hypothalamic axons emerge in response to the repelling activity of BMP7-soaked beads compared to Shh-soaked beads ($P = 0.0351$; Table 1), but that more axons appear to form axon fascicules/bundles in the presence of Shh. Our ectopic bead implant experiments indicate that Shh can induce the formation of axon fascicles and prevent axons from growing into sources of Shh protein; by contrast, hypothalamic axons simply appear to be repelled from BMP7 sources (compare Fig. 3K, L). The differences mediated by Shh and BMP7 add new insights into our understanding of axon guidance in the hypothalamus. As shown in Fig. 1, in addition to its expression in the AVM, Shh is expressed in the VZ/SVZ of the hypothalamic basal plate. Our results suggest that here, a chemorepulsive effect of Shh may repel hypothalamic axons from the VZ/SVZ of the hypothalamus and ensure that these axons travel through the mantle layer of the hypothalamus in fascicles.

4.2. A model system for hypothalamic axon guidance

Here, in vitro studies provide significant insight into the mechanisms of axon guidance within the developing chick hypothalamus. These data are consistent with the emerging idea that classical morphogens are potent axon guidance cues. Finally, our data, together with previous observations [16] suggest a model system for hypothalamic axon guidance. In this model, FGF10 is a pivotal factor that attracts both hypothalamic axons and vascular endothelial cells into the forming median eminence and neurohypophysis. Shh serves initially to induce the formation of fasciculated hypothalamic axons and directs their growth along the mantle layer, then collaborates with the chemorepellant BMP7 preventing hypothalamic neurosecretory axons from entering into other inaccurate domains of the hypothalamus, and repelling neurosecretory axons into the MVM of the hypothalamus.

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